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Influence of Silver Nanoparticles (Ag-NPs) on Bacterial Load Reduction and Some Kidney Function During Skin Wound Healing in Mice (*Mus musculus*)

M. SAEED HEYDARNEJAD[1], S. RAHNAMA[2], M. MOBINI D.[3] and P. YARMOHAMMADI[2]

[1]Research Institute of Biotechnology,
Shahrekord University, PO B 115, Shahrekord 88186, IRAN.
[2]Biology Department,
Shahrekord University, PO B 115, Shahrekord 88186, IRAN.
[3]Genetic Department,
Shahrekord University, PO B 115, Shahrekord 88186, IRAN.

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ABSTRACT

We report that silver nanoparticles (Ag-NPs) (40 nm) with minimal effect on kidney, can promote wound healing through bacterial load reduction in mice *Mus musculus*. A group of 50 BALB/c mice of about 8 weeks (weighting 24.2±3.0 g) were randomly divided into two groups: Ag-NPs and control group, each with 25 mice. A volume of 50 microliters from the nanosilver solution (10ppm) was applied to the wound bed in the Ag-NPs group while in the untreated (control) group no nanosilver solution was used but the wound area was washed by distilled water every day. The experiment lasted for 14 days until the wound was healed. The wound bacterial populations of two groups were counted separately. Further, biochemical levels of kidney including creatinine (Cr) and sodium (Na) were assessed. The results show that Ag-NPs have strong bactericidal effect on skin wound and accelerate healing and that Ag-NPs induce minimal toxicity on kidney functions as indicated by levels of Cr and Na in the serum. The obtained results of current study unequivocally confirm the role of Ag-NPs as non-toxic, antibacterial agents in all wounds and the enhancement of current prevention of infection.

Keywords: Silver nanoparticles (Ag-NPs); Mice; Creatinine (Cr); Sodium (Na); Bacterial load.

INTRODUCTION

Silver nanoparticles (Ag-NPs) are clusters of silver atoms that range in diameter from 1 to 100 nm and are attracting interest as antibacterial and antimicrobial agents for applications in medicine. They have also increasingly been used for coatings on various textiles and certain implants, for the treatment of wounds and burns, as a water disinfectant, and in air-freshener sprays[5,17]. Recent evidence suggests that Ag-NPs have potent anti-inflammatory effects[19,22,26] and accelerate wound healing[30,12].

Skin wound healing proceeds through an overlapping pattern of events including coagulation, inflammation, proliferation, matrix and tissue remodeling. The ultimate goal for wound healing is a speedy recovery with minimal scarring and maximal function[11]. For this efficient and highly controlled repair process to take place, numerous cell-signaling events are required[26].The use of antimicrobial prophylaxis is important in reducing the wound's microbial load[1]. Indeed, when associated with a heavy bacterial burden, the rate of wound healing is reduced.

The antibacterial activity of Ag-NPs could be related to several mechanisms including, induction of oxidative stress due to generation of reactive oxygen species (ROS) which may cause the degradation of the membrane structure of the cell, release of ions from the surface of nanoparticles that has been reported to cause bacterial death due to binding to cell membrane. In fact, Ag-NPs have aptly been investigated for their antibacterial property[2,18,20,23]. For instance, Kim *et al*. (2007) reported that the antimicrobial mechanism of Ag-Nps is related to the formation of free radicals and the subsequent free radical–induced membrane damage. Nevertheless, the exact mechanism of toxicity is not well understood[7].

Nanoparticles, including Ag-NPs can be transported into the blood and deposit in target organs where the nanoparticles exert potential toxic effects. Kidney could be a target because of its role in elimination of xenobiotics[21].

The kidneys regulate the amount of water and salts in the bodies by filtering the blood through millions of structures called nephrons. Creatinine (Cr) (as a by-product of muscle) is a waste product excreted through the kidneys. It is generally considered to be an accurate measurement of underlying kidney functions because it is less affected by diet, stress and dehydration. Sodium (Na) is one of the electrolytes (salts), which is essential to the functioning of the body. Na is by far the major solute in extracellular fluids, so it effectively determines the osmolarity of extracellular fluids.

There have been few studies exploring the effectiveness of Ag-NPs on skin wound healing except for some research done by Fan and Bard[9] and Hendi[11]. The objective of the study, therefore, was to gain more insight into potential influence of Ag-NPs on bacterial load reduction and some

kidney function during skin wound healing in mice *Mus musculus*.

MATERIALS AND METHODS

Mice holding

Animal test was performed with compliance of the local ethics committee. A group of 50 BALB/c mice of about 8 weeks (weighting 24.2±3.0 g) were purchased from Medical Faculty of Shahrekord University and then transferred to the laboratory. The animals were in a single group and maintained on commercial pellet diet, given deionized water *ad libitum* and kept in plastic cages in a 20±2 ◦C, 50–70% relative humidity room with a 12-h light/dark cycle. The photoperiod was provided by fluorescent tubes (Thorn, 36 W, white light), and all lighting was excluded during the scotophase. A timer was used to turn the lights on and off. After 2 weeks acclimation, the mice were randomly divided into two groups: Ag-NPs and the control group, each with 25 mice. The animals were kept fasting over night before treatment. The mice were examined daily for infections.

Preparation of Ag-NPs

Silver nanoparticles (Ag-NPs) were purchased from Nano Pars Co., Iran with a purity of 95%. The final concentration of solution was 10 ppm. The mean diameter of Ag-NPs averaged 40 nm (and ranged from 35 to 45 nm), according to the manufacturer.

Excisional wound model & Experiment

Anesthesia for experimentation was achieved with an intramuscular injection of 10 ml ketamine, 0.5 ml acepromazine, 2 ml Diazepam and about 0.5 ml Xylazine solution at a dose of 50 mg/Kg. A 2.0 × 2.0 cm2 full-thickness excisional wound was created after anesthesia. The dorsal area of each mouse was carefully shaved and the skin was disinfected with iodine. Then injury was made with some up to 10 mm in diameter, following anesthesia (Fig. 1). A volume of 50 microliters from the nanosilver solution (10 ppm) was applied to the wound bed in the Ag-NPs group daily at a given time. In the untreated (control) group no nanosilver solution was used but the wound area was washed by distilled water every day. Mice were housed separately. The experiment lasted for 14 days until the wound was healed. At the end of the experiment, the animals were then sacrificed. The blood was obtained directly from heart. The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. The biochemical levels of kidney including creatinine (Cr) and sodium (Na) were assayed by an automatic biochemical analyzer.

Bacterial load determination

To bacterial count, at fist a sterile swap was dipped into sterile normal saline, containing 0.1% (V/V) Tween 80 as detergent and then put on the distinct surface of injury in both groups. The swab was transferred to tryptone soy broth (TSB) medium and vortexed. Then, a 100 µl sample of the bacterial suspension were cultured in nutrient agar (NA), eosin-methylen blue (EMB), mannitol salt agar (MSA), and potato dextrose agar (PDA) media using surface culture method with three replicates. The incubator temperature

was set at 37°C (except for PDA that was 30°C).The total numbers of bacteria were counted carefully after completion of their growth within 24-96 hrs.

Statistic analysis

Statistical analyses were performed using Student's paired t-test, one-way ANOVA and Tukey *post-hoc* test. A p value of 0.05 was considered significant. The results showed the average value ± standard deviation.

RESULTS

Bacterial load

On day 2 of the experiment, the growth of bacteria in the culture media in mice treated with Ag-NPs was not found to be different compared with the control group. In both groups, the number of bacterial count was found to be increased on day 7 of the experiment. The observed bacterial counts (mean ± standard error) on that day were 48.0 ± 7.55 and 77.5 ± 7.5 for treatment and control groups, respectively. This increase however, was significant (t-test, $p<0.05$, Fig. 2). Indeed, Ag-NPs were found to have a significant effect on the growth of bacteria. This may show that when Ag-NPs are present on the surface of the wound area, they could more completely inhibit bacterial growth as compared with the control group.

Furthermore, as scar formation is essential in wound healing[31], scar surface area was determined. There was marked difference in the macroscopic appearance of healed wounds 14 days after wounding. The sites of the wounds treated with Ag-NPs had little scar. By contrast, the scar had not disappeared in the control group within this period. The results show that Ag-NPs have strong bactericidal effect on skin wound and accelerate healing.

Biochemical parameters

Fig. 3 demonstrates the level of Cr in both groups (treatment and control). As seen, there was some fluctuation in the level of Cr during days 7 and 14 of the experiment. However, the variation of this protein was not significant throughout the experiment ($P > 0.05$), so that the level of serum Cr tended to stay within the normal range (0.5 to 1.2 mg/dl). Regarding Na, there appeared to be little variation in the level of this electrolyte during sampling days (2, 7 and 14) and its range did not change significantly between the two groups. Taken together, the results show that Ag-NPs induce minimal toxicity on kidney functions.

DISCUSSION

The results of the current study show that silver nanoparticles (Ag-NPs) can improve skin wound healing and reduce scar appearance through bacterial load reduction and that Ag-NPs induce minimal toxicity on kidney functions as indicated by levels of Cr and Na in the serum.

Silver nanoparticles (Ag-NPs) appear to possess potent antimicrobial activity to reduce infections. In fact, Ag-NPs with strong biocidal effects are toxic to microorganisms. They can kill bacteria that cause diseases through the contamination of food, water, and wounds (Faunce and Watal,

2010). The mechanism of Ag-NPs on microorganisms is not completely clear, however, AgNPs interact with a wide range of molecular processes within microorganisms from inhibition of growth to loss of infectivity to cell death (Lara *et al*. 2011). For instance, free radicals derived from the surface of AgNPs had been suggested for the antimicrobial activity[6].

Nowadays, clinical research and therapeutic experience pay much attention to the importance of reducing the bacterial load in wounds[3]. Wound healing is an essential physiological process mediated by a variety of factors responsible for the regeneration and reorganization of damaged tissue toward its normal architecture (Han *et al*., 2012). Many factors result in delayed wound healing, one of which is infection. Bacterial infection of wound can impede the healing process (Kumar *et al*., 2010). Ag-NPs with a very large surface area are capable of releasing ionic silvers into the wound medium (Buu 2011). Thus, the bactericidal effects of Ag-NPs on skin wound in the current investigation are coincided with other studies with Ag-NPs in which a great reduction in the growth of the bacterial (as many as 16 species of bacteria) was found when using Ag-NPs[3,13,23]. Besides, in this study, with a concentration of 10 ppm Ag-NPs, a significant bacterial load reduction on skin wound was observed. The results are in agree with other studies in which Ag-NPs exhibit strongest toxicity effect within a range of 10-50 ppm[14]. In fact, Ag-NPs at doses below levels that cause argyria or argyriosis are generally considered to be relatively non-toxic[28]. In addition, scar appearance and the extent of inflammation at the wound site was decreased in the mice treated with AgNPs so that the wound area was little and transient compared with the control group. Similarly, Tian[26] in investigating the wound-healing properties of Ag-NPs found rapid healing and improved cosmetic appearance in an animal model. They then claimed that through their antimicrobial properties, Ag-NPs exert positive effects. Typically, the wound healing involves steps that include inflammation around the site of injury, angiogenesis and the development of granulation tissue, repair of the connective tissue and epithelium, and ultimately remodeling that leads to a healed wound (Atiyeh *et al*., 2007). Ag-NPs have useful anti-inflammatory effects and improve wound healing[4]. For example, Wright *et al*.[29] have proved that Ag-NPs significantly suppress inflammatory cytokines and induced apoptosis of inflammatory cells. This is because some studies have demonstrated that a number of cytokines are involved in the progression of the wound healing[8,16].

Cr is a break-down product of creatine phosphate in muscle, and is usually passed into the bloodstream and then out in urine. Measuring serum Cr is the most commonly used indicator of renal function[25]. A high blood level of Cr indicates that the kidneys may not be functioning properly. Furthermore, GFR (glomerular filtration rate) as the best overall biomarker of kidney function in mice is determined by assessing serum Cr[24]. As no significant increase in the serum Cr was observed in the current study consequently the mice kidney treated with Ag-NPs were not affected.

Similarly, the kidney plays a critical role in maintenance of ECF volume, via Na. Na concentration is inextricably linked with extracellular fluid (ECF) concentration; therefore interpretation of sodium levels should always include consideration of the hydration status of the patient. Thus, a high blood sodium level in the control group of the present study may be due to inadequate water intake and dehydration, as no such occurrence took place for the group of mice treated with Ag-NPs (Wardener *et al*., 2004).

Changes of serum biochemical parameters including Cr and Na of the present study indicated that the kidney was not significantly affected in mice treated with 10 ppm of Ag-Nps. The results are in complete agreement with other studies[9,11] showing that Ag-Nps exert positive effects in skin wound healing with minimal toxicity on kidney functions.

Taken together, the obtained results of the current study show that Ag-NPs are potential candidates of strong antibacterial activity on skin wound which accelerate healing and that they have minimal effect on kidney function.

Fig. 1. A 2.0 × 2.0 cm^2 full-thickness excisional wound after shaving the dorsal area of each mouse, using anesthesia.

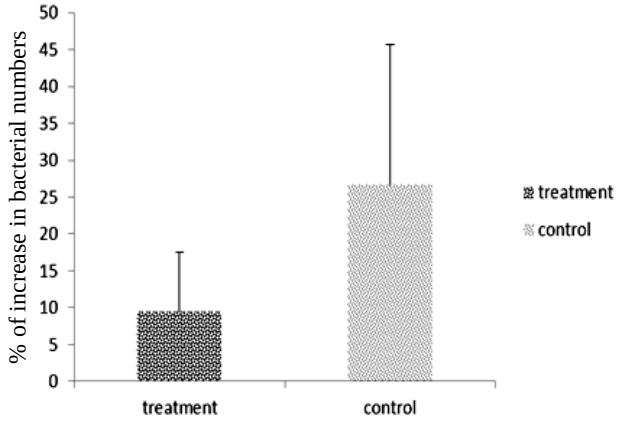

Fig. 2. Number of bacteria as a function of the Ag-NPs expressed as a percentage of the number of colony-forming units (CFU) grew up on TSB agar plates containing 10ppm of Ag-NPs (treatment) and no tretament (control) for day 7 of the experiment.

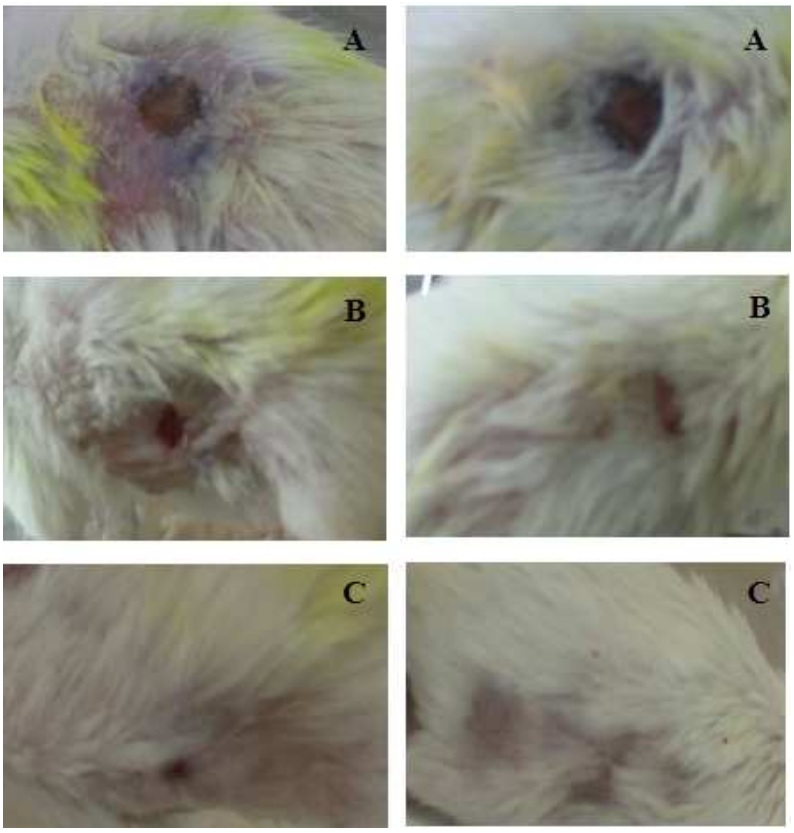

Fig. 2. Ag-NPs accelerate skin wound healing and achieve superior cosmetic outcome. Time taken for skin wounds to heal in mice treated with Ag-NPs (right pictures) as compared with no treatment (left pictures).

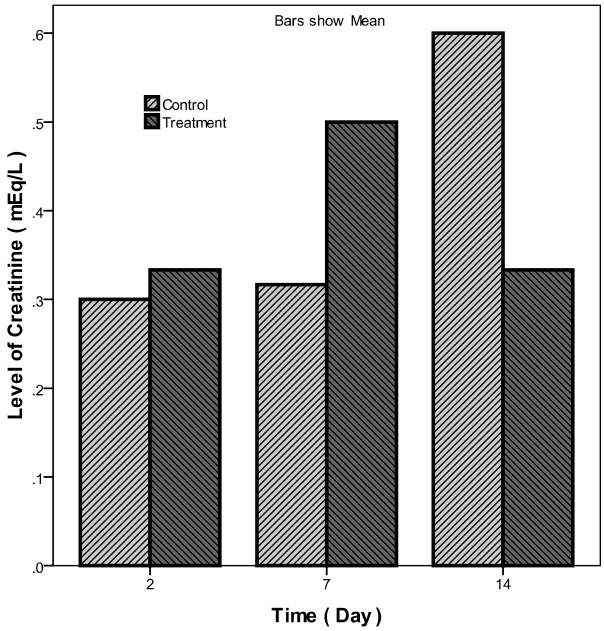

Fig. 3. The serum levels of sodium (above) and serum Cr (down) in mice treated with Ag-NPs (treatment) and without Ag-NPs (control) during days 2 through 14 of the experiment.

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